The Development of a Markerless Deletion System in *Desulfovibrio vulgaris* Hildenborough

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ESPP2 Environmental Stress Pathway Project

DOE GENOMICS:GTL ACCELERATING DISCOVERY FOR ENERGY AND ENVIRONMENT — OFFICE OF SCIENCE, U.S. DEPARTMENT OF ENERGY

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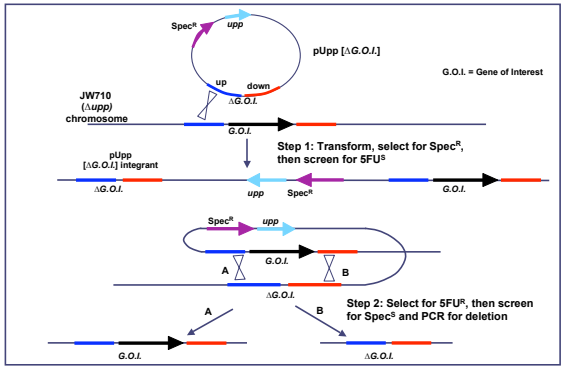
Abstract

To fully explore microbial community dynamics, stability of the composition with time and changing nutritional and environmental factors must be explored. In order to confirm sources and sinks of metabolites, both during degradation and biosynthesis, it would be most useful to create pivotal deletions in various members of the community. We are pursuing genetic tools that can possibly be applied to strains with limited genetic accessibility. These tools are being developed in *Desulfovibrio* and include an inframe deletion procedure and plasmid modification in extracts to facilitate genetic exchange processes.

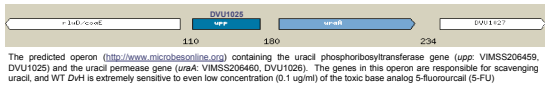
To properly study metabolic pathways, it is necessary to delete several genes that may have compensatory activities. Our model system, the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough has seen enormous progress in genetic manipulation; however, the current deletion method of marker exchange mutagenesis does not allow for easy selection of multiple sequential gene deletions because of the low number of selectable markers available in *D. vulgaris*. To broaden the repertoire of genetic tools available for manipulation in *D. vulgaris*, an in-frame markerless deletion system is being developed based on the *upp*-encoded uracil phosphoribosyltransferase as an element for a counterselection strategy. In wild-type *D. vulgaris*, growth is inhibited by the toxic pyrimidine analog 5-fluorouracil (5-FU), whereas a mutant bearing a deletion of the *upp* gene is resistant to 5-FU. The introduction of a plasmid containing the wild-type *upp* gene expressed constitutively from the *aph*(5′)–III promoter (the promoter for the kanamycin resistance gene in Tn5) into the *upp* deletion strain restored sensitivity to 5-FU. This observation is the basis for the establishment of a two-step integration and excision strategy for the deletion of genes of interest. Since this deletion does not contain an antibiotic cassette, multiple gene deletions can be generated in a single strain using this method.

This in-frame markerless deletion method is currently being evaluated through the construction of a deletion of the putative formate dehydrogenase alpha- and beta- subunits, DVU0587 and DVU0588. In addition, Gateway Technology methods are being developed that would expedite the process of generating the required deletion vectors by the construction of a destination vector containing the constitutively expressed wild-type *upp* gene. This new method is being utilized to generate a deletion for the R-subunit (DVU1703) of a type I restriction-modification system.

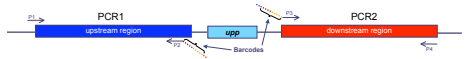
Markerless Deletion Strategy



Construction of JW710 (Δ*upp*) Strain



The predicted operon (http://www.microbesonline.org) containing the uracil phosphoribosyltransferase gene (*upp*: VIMSS206459, DVU1025) and the uracil permease gene (*uraA*: VIMSS206460, DVU1026). The genes in this operon are responsible for scavenging uracil, and WT *Dv*H is extremely sensitive to even low concentration (0.1 ug/ml) of the toxic base analog 5-fluorouracil (5-FU)



Regions upstream and downstream from *upp* gene were amplified from WT *Dv*H via two, separate PCR experiments. The primers (P2 and P3) were designed to contain two barcodes to be introduced on the end of each region, as well as containing overlapping regions.

A third PCR experiment was used to join the products from PCR1 and PCR2 together to form the *upp* deletion construct. The deletion construct was ligated into the pBluescript plasmid, the sequences verified, and the plasmid named pMO711.

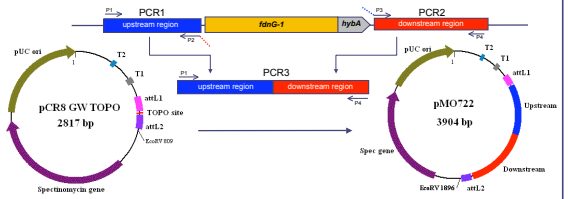
The plasmid pMO711 was then electroporated into WT *Dv*H. Cells that had successfully integrated the deletion construction were selected by growing the cells on LS4 medium containing 40 ug 5-FU/ml.

Several isolated, single colonies were screened for the deletion using Southern blot analysis. One of the isolates was chosen and named JW710

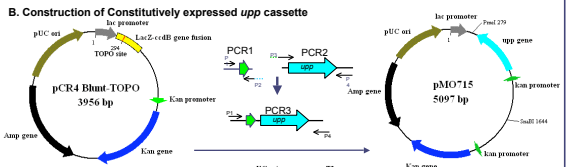
Construction of DVU0587 Markerless Deletion Vector

A. Deletion Cassette Construction (Δ*fdnG-1*)

Regions upstream (~500 bp) and downstream (~600 bp) of the formate dehydrogenase genes *fdnG-1* (VIMSS20953, DVU0587) and *hybA* (VIMSS209532, DVU0588) genes were amplified from WT *Dv*H via two, separate PCR experiments. A third PCR experiment was used to join PCR1 and PCR2 products together to form the in-frame markerless deletion construct, PCR3. The in-frame markerless deletion construct was ligated into the pCR8 GW TOPO plasmid, the sequences verified, and the plasmid named pMO722.

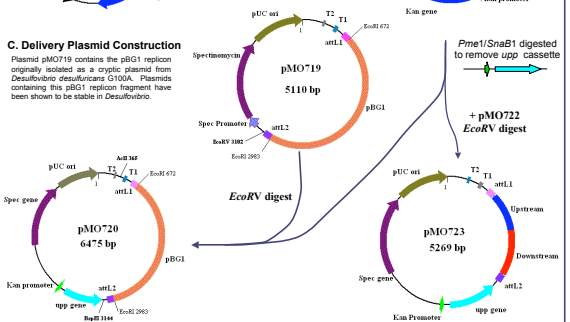


B. Construction of Constitutively expressed *upp* cassette

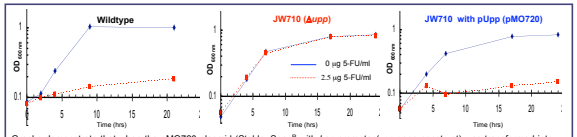


C. Delivery Plasmid Construction

Plasmid pMO719 contains the pBG1 replicon originally isolated as a cryptic plasmid from *Desulfovibrio desulfuricans* G100A. Plasmids containing this pBG1 replicon fragment have been shown to be stable in *Desulfovibrio*.



Confirmation *Kan* promoted *upp* gene is Functional



Graphs demonstrate that when the pMO720 plasmid (Stable, Spec^R with *kan* promoter/*upp* gene construct) was transformed into strain JW710 (Δ *upp*) sensitivity to 5-FU was restored to wild-type levels.

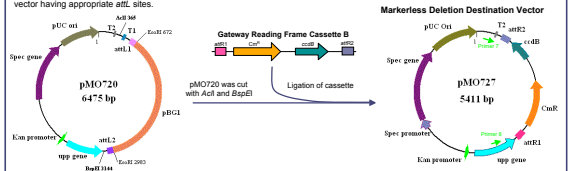
Results of DVU0587 Markerless Deletion

After verifying the *kan* promoter/*upp* gene was able to restore sensitivity, pMO723 was electroporated into JW710 and integration of this plasmid into the chromosome confirmed To date, nearly 200 individual 5-FU^R and Spec^S isolates have been screened as possible markerless deletion mutants: however, all isolates were determined to have recombined back to wildtype (Step 2A in Markerless deletion strategy), which was inferred as indicating that DVU0587 and/or DVU0588 may be essential.

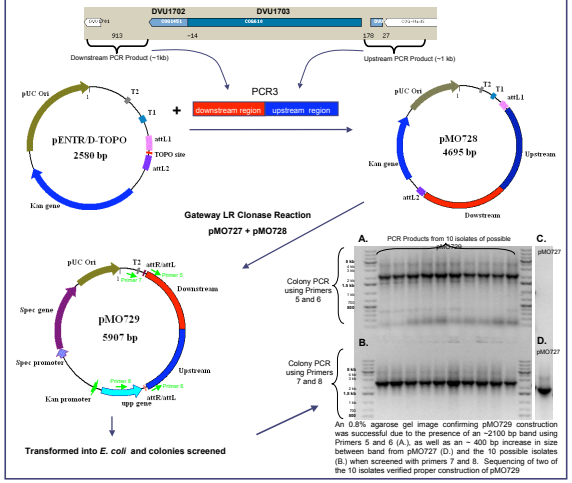
GatewayTechnology

A. Construction of Inframe Markerless Deletion Destination Vector

Using the Gateway Vector Conversion System (Invitrogen), the Gateway Reading Frame B (1713 bp) was ligated and then transformed into *ccdB* Survival Cells (Invitrogen) by selecting for Chloramphenicol and sequencing to verified proper construction of pMO727. The bacteriophage λ–derived *attR* sites in the cassette will allow recombinational cloning of markerless deletion constructs from an entry clone vector having appropriate *attL* sites.



B. Entry clone construction (eg: ΔCOG610, type I restriction-modification enzyme, R subunit [DVU1703])



Conclusions

- ***upp* provides a useful counterselection marker in *Desulfovibrio vulgaris* Hildenborough**
- **Vectors for inframe/markerless deletions have been constructed and confirmed**
- **Multiple mutations can now be generated in *Desulfovibrio vulgaris* Hildenborough**
- ***fdnG*1 and/or *hybA* may be essential in *Desulfovibrio vulgaris* Hildenborough**

ACKNOWLEDGEMENT

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